


Loss of pollen-specific phospholipase NOT LIKE DAD triggers gynogenesis in maize

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Abstract

Gynogenesis is an asexual mode of reproduction common to animals and plants, in which stimuli from the sperm cell trigger the development of the unfertilized egg cell into a haploid embryo. Fine mapping restricted a major maize QTL (quantitative trait locus) responsible for the aptitude of inducer lines to trigger gynogenesis to a zone containing a single gene *NOT LIKE DAD* (*NLD*) coding for a patatin-like phospholipase A. In all surveyed inducer lines, *NLD* carries a 4-bp insertion leading to a predicted truncated protein. This frameshift mutation is responsible for haploid induction because complementation with wild-type *NLD* abolishes the haploid induction capacity. Activity of the *NLD* promoter is restricted to mature pollen and pollen tube. The translational *NLD*::citrine fusion protein likely localizes to the sperm cell plasma membrane. In *Arabidopsis* roots, the truncated protein is no longer localized to the plasma membrane, contrary to the wild-type *NLD* protein. In conclusion, an intact pollen-specific phospholipase is required for successful sexual reproduction and its targeted disruption may allow establishing powerful haploid breeding tools in numerous crops.

Keywords embryo; fertilization; gynogenesis; haploid; phospholipase; *Zea mays*

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Plant Biology

DOI 10.15252/embj.201796603 | Received 26 January 2017 | Revised 8 February 2017 | Accepted 9 February 2017 | Published online 22 February 2017

The EMBO Journal (2017) 36: 707–717

See also: **D Jackson** (March 2017)

Introduction

The mixing of male and female genetic information during sexual reproduction is considered as key to the evolutionary success of

higher eukaryotes and is the basis of plant breeding. At the same time, asexual reproduction occurs in nature and is exploited in breeding to fix interesting genetic combinations. One form is gynogenesis, in which an unfertilized egg cell develops into a haploid embryo upon stimulation by a male gamete. After spontaneous or artificial chromosome doubling, diploid plants can be obtained, which are completely homozygous and reflect a fixed state of the mother plant. Such doubled haploids are routinely used in plant breeding to simplify logistics and reduce time to market. Consequently, the elucidation of the molecular mechanisms triggering gynogenesis represents a major scientific and economic interest (Geiger & Gordillo, 2009).

In maize, spontaneous gynogenesis is very rare with frequencies in the 0.1% range (Chase, 1949). Beyond genetics, stress treatments such as strong heat shock (Randolph, 1932), X-radiation, or UV light (Mathur *et al*, 1980) have been reported to influence haploid induction rates in maize (Chase, 1949). Much higher frequencies suitable for hybrid breeding are achieved by pollination with the so-called inducer lines, which all seem to have stock 6 (Coe, 1959) as common ancestor (Weber, 2014; Hu *et al*, 2016). The haploid induction rate is determined mainly by the genetic constitution of the inducer line but also by that of the female line receiving inducer pollen (Chase, 1969). Eight paternal quantitative trait loci (QTL) were uncovered in inducer lines (Barret *et al*, 2008; Prigge *et al*, 2012) and two maternal QTLs in receiving females (Wu *et al*, 2014). Among the paternal QTLs, the one located on chromosome 1 and called successively *gynogenesis inducer1* (*ggi1*) (Barret *et al*, 2008) and *quantitative haploid induction rate 1* (*qhir1*) (Prigge *et al*, 2012) has by far the strongest effect explaining in certain crosses up to 66% of the genotypic variance for haploid induction. The *ggi1/qhir1* locus is supposed to be mandatory for haploid induction ability, and has been associated with poor transmission of inducer pollen leading to segregation distortion (Barret *et al*, 2008; Prigge *et al*, 2012). The *ggi1/qhir1* locus was fine-mapped to a region of 243 kb on chromosome 1 (Dong *et al*, 2013). A second important QTL, named *qhir8*, was found to explain 20% of the genotypic variance and

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to be important for enhancement of the inducing capacity of *ggi1/qhir1*. The *qhir8* locus was fine-mapped to a region of 789 kb on chromosome 9 (Liu *et al*, 2015).

It has been challenging to elucidate the mechanisms underlying gynogenesis induced by stock 6 and its derivatives for two reasons: Firstly, the partial nature of the induction makes it difficult to predict for a given pollen tube/embryo sac interaction, whether it will result in gynogenesis or not. Secondly, comparative studies between inducer and non-inducer lines are hampered by the heterogeneity of the genetic background, since no near-isogenic material is available due to the polygenic nature of the induction capacity. Consequently, it is not surprising that some of the published results remain contradictory. In inducer lines, all steps up to the development of the trinucleate pollen are normal, since there are no biologically relevant differences in the number of nuclei between inducer and control lines, neither in mature pollen nor in germinated pollen tubes (Mahendru & Sarkar, 2000). First differences appear during pollen tube germination since pollen from non-inducer lines germinates faster than pollen from inducer lines and outcompetes pollen from inducer lines in successive pollination experiments (Xu *et al*, 2013). At the level of the embryo sac, a cytological study detected remnant sperm cell nuclei after pollination with inducer pollen but not with wild-type pollen (Swapna & Sarkar, 2012) and molecular studies even revealed remnants of paternal genetic material in haploid embryos (Zhao *et al*, 2013). Finally, the presence of haploid embryos on ears pollinated by an inducer line is clearly correlated with a general decrease in kernel number and an increase in the number of defective kernels (Xu *et al*, 2013).

Phospholipases (PLs) are enzymes that cleave phospholipids, the major component of all cellular membranes. The hydrolysis site within the phospholipid molecule defines four PL types. Whereas PLAs and PLBs remove hydrophobic fatty acid tails from the glycerol backbone, PLCs and PLDs remove either the hydrophilic phosphate head or side chains attached to it (Wang, 2001; Park *et al*, 2012; Vines & Bill, 2015). PLs have broad and diverse roles, and it is now clear that they have both structural and signaling functions: PL enzymes take part in lipid metabolism and membrane remodeling but also generate lipid-derived messengers involved in membrane associated lipid signaling pathways that regulate multiple cellular processes (Wang, 2001; Park *et al*, 2012). Within the PL family, patatin-like PLs represent a group of proteins found in diverse organisms (bacteria, yeast, plant, and animal) that share high similarity to the catalytic domain of patatin, an abundant protein of the potato tuber (Scherer *et al*, 2010). This catalytic center consists of the esterase box GX SXG and the phosphate- or anion-binding element DGGGXXG. Patatin-like PLs preferentially hydrolyze fatty acids in the sn-2 position (Banerji & Flieger, 2004; Scherer *et al*, 2010).

Pollen development is one of the processes for which PL activity is essential. In *Arabidopsis*, three *PLA2* genes were found to be expressed in mature pollen, and knockdown of their expression by RNAi results in pollen lethality (Kim *et al*, 2011). On the subcellular level, these three *PLA2* enzymes located to the endoplasmic reticulum and/or Golgi (Kim *et al*, 2011). In addition, mutants in the chloroplast-localized *PLA DAD1* (DEFECTIVE IN ANTHHER DEHISCENCE1) are impaired in anther dehiscence and show defects in pollen grain maturation (Ishiguro *et al*, 2001). Earlier aberrations in pollen development as well as defects in the female gametophyte were observed in the *plc2* mutant of *Arabidopsis* (Li *et al*, 2015).

This work presents the positional cloning of the *ggi1/qhir1* QTL and provides evidence that a mutation in the patatin-like phospholipase A NOT LIKE DAD (NLD) is the key factor triggering gynogenesis in maize after pollination by inducer lines.

Results

Fine mapping of the *ggi1/qhir1* locus

The starting points for the fine mapping of the *ggi1/qhir1* locus were the creation of 96 highly recombinant inbred lines (F2i3S2) and 18 single-seed descendants (selected for induction over seven generations) of a cross between the inducer line PK6 and the inbred line DH99 (Barret *et al*, 2008). Based on haploid induction tests and mapping with 10 markers in the *umc1144/bnlg1811* interval (Fig 1A), the two inducer lines with the shortest PK6 genome region were backcrossed to the recurrent DH99 parent. In parallel, the use of additional markers narrowed the confidence interval down to the region between markers SYN24144 and PZE-101081844 (Fig 1A and Appendix Table S1). After selfing plants derived from the backcrosses heterozygous at the locus, 10,275 plantlets were screened for new recombination with markers GRMZM2G100497_10, GRMZM2G152877_6, and SYN35770 (Fig 1A and Appendix Table S1) located outside of the redefined QTL interval but inside the original breakpoints; 531 recombinant plants were obtained.

The phenotypic characterization of the new recombinants exploited the strong correlation between haploid induction rate and segregation distortion (Barret *et al*, 2008; Prigge *et al*, 2012; Xu *et al*, 2013). For a subset of 48 recombinants with breakpoints in the redefined confidence interval, the segregation distortion was determined by genotyping for each recombinant a small population obtained by self-pollination with 16 markers. The strongest segregation distortion was observed with marker GRMZM2G471240_1 (Fig 1B and Appendix Table S1).

Among the 48 families, 28 contained plants homozygous for the recombinant allele. This subset was used as pollen parents to assess their haploid induction rate in test crosses (Fig 1D). The results indicated that the locus responsible for haploid induction was located between markers GRMZM2G471240_13 (breakpoint of the recombinant named 71-19-29) and 155K10_55 (breakpoint of recombinants named 71-39-162 and 71-29-69) in a 166-kb interval on the B73 reference genome (Fig 1D and Appendix Table S1).

Structure of the confidence interval in the PK6 genome

The occurrence of 30 consecutive dominant PAV (Presence–Absence Variant) markers (absence in PK6 genome) between positions 68.24 Mbp and 68.36 Mbp of the B73 reference genome was a strong indication for important structural differences between the two genomes (Appendix Table S1). Consequently, it was necessary to determine the DNA sequence of the corresponding genomic interval in the genotype PK6 to assess the number and type of genes predicted at the *gim1/qhir1* locus. Thus, a BAC library was created from PK6 gDNA. The alignment of the consensus sequence of five overlapping BAC clones (GenBank accession KX852318) with the B73 reference genome revealed the replacement of a 156-kb region of B73 by an unrelated sequence of 70 kb in PK6, explaining the

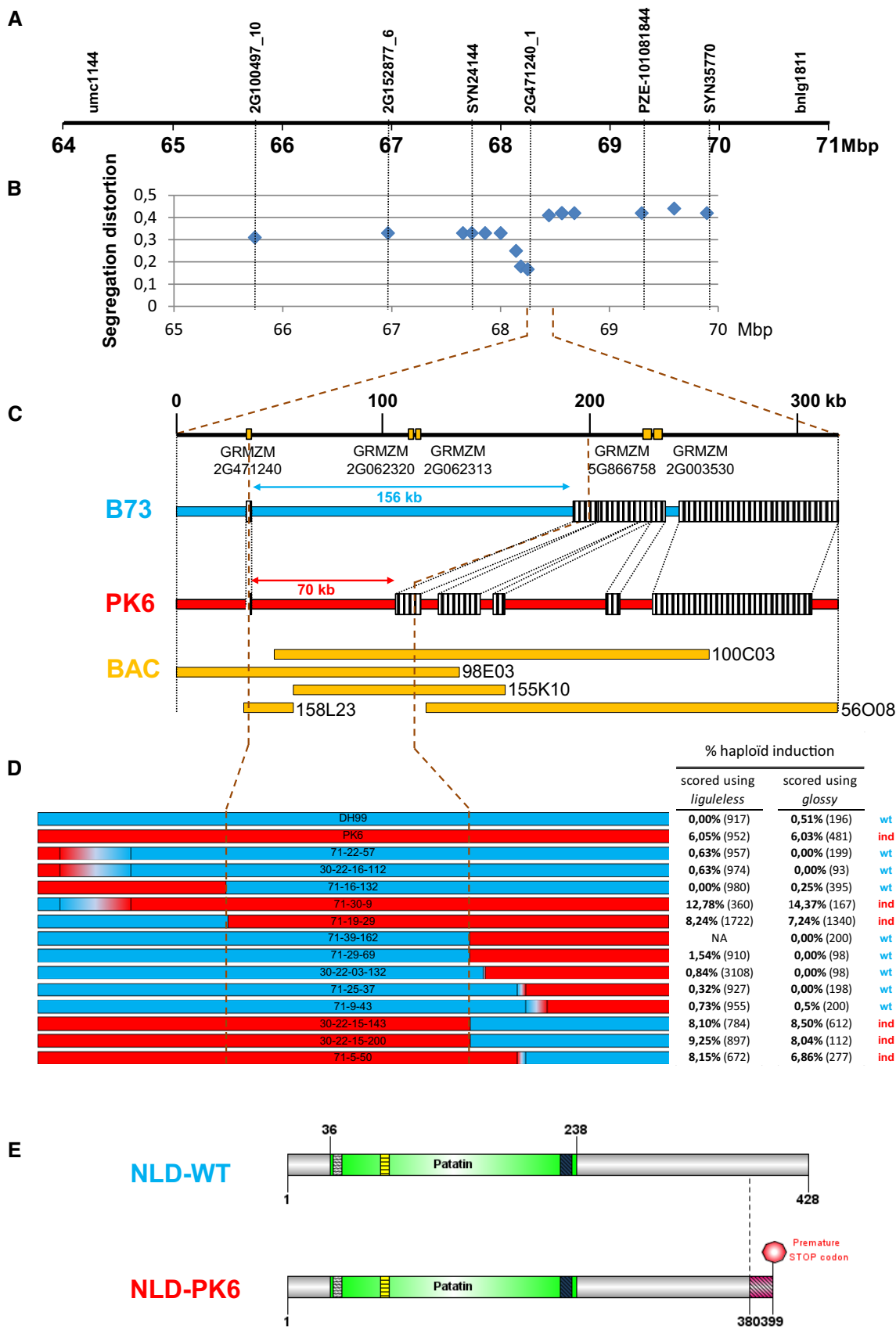


Figure 1. Fine mapping and structure of the *ggi1/qhir1* locus.

- A Key markers used during fine mapping. The scale refers to the reference sequence of chromosome 1 (RefGen_v3).
- B Segregation distortion at the *ggi1/qhir1* for 16 markers (blue losanges) expressed as number of PK6 alleles divided by number of DH99 alleles and positioned on the same interval as described in (A).
- C GEvo analysis (<https://genomevolution.org/coge/GEvo.pl>) of the B73 and PK6 sequences. Regions with more than 95% similarity are marked by hatched boxes and linked by dotted lines. Gene models predicted in the B73 sequence are labeled with their GRMZM identifiers. Brown dashed lines delimit a 166-kb region of B73 and a 80-kb region of PK6. Both regions have only 10 kb in common and arrows indicate the 156-kb region of B73 replaced in PK6 by a non-related 70-kb region. BAC clones used to establish the PK6 sequence are shown as yellow boxes below the PK6 sequence.
- D Schematic drawing of key recombinants using the B73 sequence as reference. Genome regions stemming from PK6 and DH99 are colored in red and blue, respectively. A color transition was chosen if the distance between the discriminating markers was more than 1 kb. The percentage of haploid induction of the corresponding plants is indicated as % (number of offspring analyzed) and classed as ind (inducer) or wt (wild-type = non-inducer).
- E Structure of predicted NLD proteins. Both wild-type and NLD-PK6 contain a patatin/phospholipase domain represented in green. The light gray box represents the phosphate/anion-binding elements, the yellow horizontally hatched box the esterase box, and the dark box the catalytic dyad-containing motif. Due to a frameshift, NLD-PK6 lacks the 49 C-terminal amino acid residues in comparison with wild-type protein, which are replaced by 20 non-related amino acid residues (positions 380–399 represented by red hatched box) ending with a premature STOP codon.

absence of additional recombination between the breakpoints defining the 166-kb interval in B73, which was reduced to 80 kb in PK6 (Fig 1C). It also revealed the absence of genes GRMZM2G062320 and GRMZM2G062313 in the PK6 sequence. In addition, several other insertions or replacements in the PK6 genome existed in the proximity of the *gim1/qhir1* locus (Fig 1C).

Ab initio analysis predicted 13 genes in the 80-kb interval of genotype PK6 (Appendix Table S2). Ten of them carried annotations related to retrotransposon families with multiple copies across the genome. Two others genes, FGENESH_18 and FGENESH_19, had more than 10,000 and 182 copies in the B73 reference genome, respectively. The only single copy gene was FGENESH_8 (GRMZM2G471240) annotated as patatin-like phospholipase, which was the only functional annotation not related to retrotransposons among the 13 genes. In comparison with B73 and DH99, the PK6 allele carried a 4-bp insertion in exon 4 corresponding to marker GRMZM2G471240_14. A survey of this marker in a genetic diversity panel of 92 lines and a collection of 12 haploid inducer lines (including PK6, the widely used RWS and the initial stock 6) detected the PK6 insertion in all inducer lines but in none of the lines of the diversity panel, whereas other polymorphisms of GRMZM2G471240 were not restricted to inducer lines (Appendix Table S3). Thus, the 4-bp insertion represented not only a discriminant marker for haploid induction but also its possible cause. Reinforcing this idea, lines possessing the same haplotype as PK6 in the corresponding interval and differing only by the 4-bp insertion (EM1201, Nys302, Mo1W, and Tx303) were not able to induce haploids (Appendix Table S3). This result ruled out a direct role of the two candidates, GRMZM2G062320 and GRMZM2G062313, in haploid induction. The sum of the mapping and sequencing data identified GRMZM2G471240 as the only low copy gene candidate in the PK6 80-kb confidence interval for haploid induction. This candidate gene was named *NOT LIKE DAD* (*NLD*) because the PK6 allele induces maternal embryos without paternal contribution. Parallel independent studies named the same gene *MATRILINEAL* (*MTL*) and *Phospholipase A1* (*ZmPLA1*) (Kelliher et al, 2017; Liu et al, 2017).

NLD codes for a patatin-like phospholipase expressed in pollen

The analysis of the deduced amino acid sequence of NLD was based on transcript variant GRMZM2G471240_T01 (Appendix Fig S1), in which the second of the four exons was larger than in variant GRMZM2G471240_T02. A single RT–PCR band of the size expected

for variant T01 indicated that this variant was the main transcript in maize. The deduced NLD amino acid sequence contained a patatin/phospholipase domain (IPR002641, Fig 1E), and phylogenetic analysis of maize and *Arabidopsis* patatin-like PLA placed NLD among class II patatin-like PLA (Appendix Fig S2). The 4-bp insertion in NLD exon 4 found in haploid inducer lines was also found in cDNA. This 4-bp insertion causes a frameshift that replaces the last 49 amino acids of the wild-type protein by an unrelated amino acid sequence of 20 aa followed by a premature STOP codon (Fig 1E and Appendix Fig S3).

NOT LIKE DAD expression in the maize plant was restricted to the mature stage of the male reproductive organ (tassel) as revealed by gene-specific qRT–PCR experiments (Fig 2A), in agreement with public microarray data (Appendix Fig S1). Compared to the non-inducer line DH99, the inducer PK6 line showed slight but significant increase of *NLD* expression in mature tassel (Fig 2B). In light of the high and specific expression of *NLD* in mature tassel, it seemed interesting to increase the spatial resolution and to distinguish between sporophytic (anther) and gametophytic (pollen) expression. A 2.6-kb upstream fragment from genotype B73 was transcriptionally fused to the *GUS* reporter gene (*pNLD::GUS*) and used for maize transformation. *GUS* staining was found in pollen grains whereas it was absent in anther tissues (Fig 2C). In a time course experiment during pollen development, the onset of *NLD* promoter activity coincided with the second pollen division, which leads to tricellular pollen with sperm cells (Fig 2D and E), specifying an expression of *NLD* in mature pollen. After pollination of wild-type plants with transgenic *pNLD::GUS* pollen, observation of germinating pollen, silks, and ovules 17 h after pollination (HAP) showed *GUS* staining in pollen tube and at the interface of pollen tube and ovule (Fig 2F). Some *GUS* staining was still observed at 48 HAP, but disappeared at 72 HAP (Fig 2F). Even if the important half-life time of the *GUS* protein makes precise statements impossible, these results suggest that the *NLD* promoter is active during the entire fertilization process.

Wild-type *NLD* abolishes the haploid induction phenotype of the inducer line

To test the hypothesis that the 4-bp insertion in *NLD* was responsible for haploid induction, complementation of the mutation was carried out in an A188 genetic background. This choice was motivated by the fact that A188 represents one of the rare maize inbred lines that can be readily transformed. In a first step, the PK6 *NLD* locus was introgressed over four generations into A188 to create a

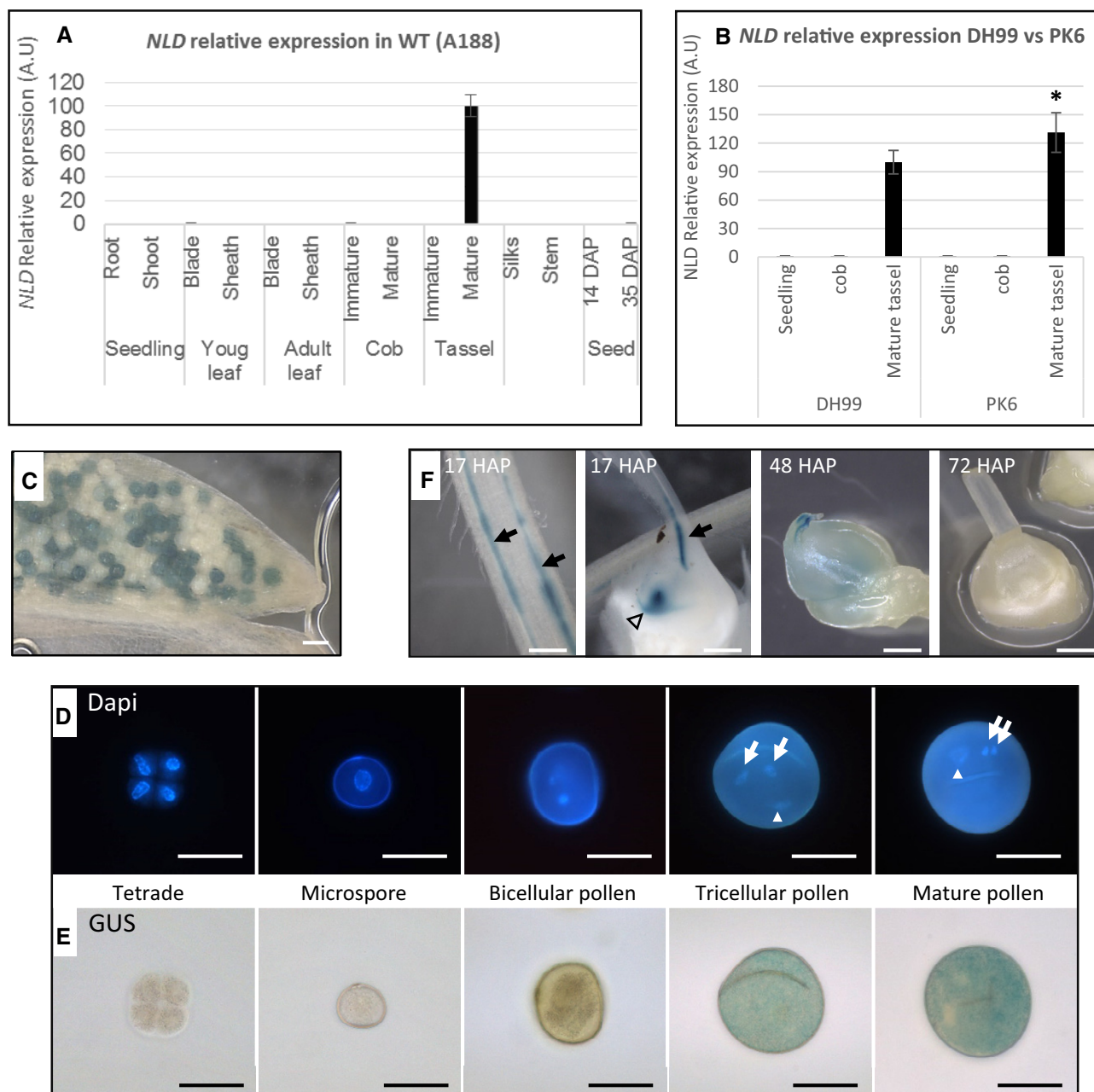


Figure 2. Pollen-specific expression of *NLD* and temporal analysis of *NLD* promoter activity.

A, B Relative *NLD* expression levels in major tissues of wild-type line A188 (A) and in selected tissues of wild-type line DH99 and inducer line PK6 (B) were determined by qRT-PCR. Values are means of at least three biological replicates \pm SE. Asterisks (*) denote significant differences (P -value < 0.05 ; t -test).

C–F Temporal analysis of *NLD* promoter activity at different stages of pollen development visualized by histochemical detection of the GUS reporter. (C) In mature anthers of hemizygous plants, blue GUS staining was found in about 50% of the pollen grains, whereas no GUS staining was observed in the anther. (D) Determination of pollen stages by visualization of nuclei by DAPI (4',6-diamidino-2-phenylindole) fluorescence. (E) Bright field of the same pictures as in (D), showing GUS staining only in pollen grain at the tricellular stage when the sperm cells (white arrow) and vegetative cell (white arrow head) are present. (F) Observations of wild-type silk at 17 h after pollination (HAP), and wild-type ovules at 17 HAP, 48 HAP, and 72 HAP of plants crossed with transgenic *pNLD::GUS* pollen, revealed GUS activity in the pollen tube (black arrows) and in the embryo sac (arrow head) at 17 HAP, whereas no GUS staining was observed at 72 HAP. Scale bars, 50 μ m (D, E) or 100 μ m (C, F).

near-isogenic inducer line (A188^{NLD-PK6}). The haploid induction rate of the successive backcrosses dropped from 3.59% in BCOS1 to 0.50% in BC3S1 (Table 1), confirming the need of other favorable loci for enhanced haploid induction capacity (Prigge *et al*, 2012; Liu

et al, 2015). This rather low induction rate remained significant because A188 was a non-inducer line with an extremely low spontaneous haploid induction rate (0 haploids out of 4,487 seedlings tested, Table 1). In parallel, A188 was transformed with a genomic

Table 1. Haploid induction rate of inbred line A188 with *NLD-PK6* introgressions and *NLD-DH99* complementation.

Lines tested	<i>NLD</i> locus	Haploid induction rate (haploids/offspring analyzed)
A188 (WT)	A188/A188	0% (0/4487)
BC0S1 A188 ^{NLD-PK6}	PK6/PK6	3.59% (18/501)
BC2S1 A188 ^{NLD-PK6}	PK6/PK6	0.81% (17/2099)
BC3S1 A188 ^{NLD-PK6}	PK6/PK6	0.5% (4/802)
BC3S1 A188 ^{NLD-PK6} + <i>NLD-WT</i>	PK6/PK6 + DH99/DH99	0% (0/606)

version of *NLD* from the non-inducer line DH99 under the control of the constitutive rice *Actin* promoter. The resulting *NLD*_{DH99}-OE line, which showed a ninefold increase in *NLD* transcript accumulation in pollen compared to A188 wild type (Appendix Fig S4), was crossed with A188^{NLD-PK6}. No haploid induction was observed with plants homozygous for both the *NLD-PK6* mutation and the *NLD*_{DH99}-OE T-DNA cassette (Table 1), demonstrating that the presence of wild-type *NLD* abolished the A188^{NLD-PK6} inducer capacity. These data led to the conclusion that the PK6 allele of *NLD* was responsible for maternal haploid induction at the *gim1/qhir1* locus.

Truncation of *NLD* protein leads to loss of plasma membrane localization

To investigate the mechanism by which the truncation of *NLD* interfered with double fertilization, the subcellular localization of both the full-length wild-type *NLD* and the truncated *NLD-PK6* was examined. C-terminal fusions with CITRINE fluorescent protein were introduced under the control of the constitutive *UBI10* promoter in *Arabidopsis* (Fig 3A–D). Confocal imaging of roots showed a clear plasma membrane localization together with localization in cytoplasm and in endomembrane compartments for *NLD*::CITRINE (Fig 3A and B). In contrast, *NLD-PK6*::CITRINE signal was absent in the plasma membrane and observed exclusively in cytoplasm and to a lesser extent in endomembrane compartments (Fig 3C and D). This result suggested that the truncated *NLD-PK6* was mis-localized and its absence in the plasma membrane was probably the cause for haploid induction underlying the *gim1/qhir1* locus in the PK6 inducer line.

To address the subcellular expression pattern in maize pollen, both wild-type *NLD* and truncated *NLD-PK6* were expressed under the *NLD* promoter. Confocal imaging of mature pollen and germinating pollen tubes showed a clear fluorescence signal in sperm cells for full-length wild-type protein, whereas only background autofluorescent signal was observed for the truncated protein (Fig 3E–H and Appendix Fig S5). The rather small size and the high nucleus/cytoplasm ratio of sperm cells hampered subcellular observation with conventional confocal microscopy. The use of structured illumination microscopy (SIM) allowed to localize the wild-type *NLD*::citrine signal to what appears to be the plasma membrane of the sperm cells, confirming the results observed in *Arabidopsis* roots (Fig 3I and Appendix Fig S5D). An *in silico* analysis of the wild-type *NLD* protein sequence predicted the absence of a signal peptide but the presence of an S-palmitoylation site at C10 and an S-palmitoylation or S-farnesylation site at C423 (Xie *et al*, 2016) (Appendix Fig

S6). The presence of two lipid anchor sites was coherent with a localization in the plasma membrane and the loss of the C-terminal lipid anchor in the truncated *NLD-PK6* protein provided a plausible explanation of its mis-localization.

Discussion

Structural rearrangements at the *gim1/qhir1* locus between inducer lines and the reference genome

Fine mapping of the *gim1/qhir1* locus identified *NLD* as the only low copy gene with a functional annotation not related to retrotransposons in the 80-kb confidence interval of the inducer line PK6. The candidate genes GRMZM2G062320 and GRMZM2G062313 present in the B73 reference genome, but absent in PK6 interval, can be ruled out because lines with similar haplotype missing these two genes are not able to induce haploids. This makes *NLD* a prime candidate to be responsible for both haploid induction and segregation distortion. More precisely, the left border of the confidence interval is determined by two recombinants within *NLD*, reducing the causal region to the 3' end of the gene. A 4-bp insertion in the last exon of the inducer allele is likely the causal polymorphism, since it is found in all surveyed inducer lines and absent in a panel of lines representing the genetic diversity of maize. In particular, lines EM1201 and Nys302, which have the same haplotype at the *gim1/qhir1* locus as the inducer line PK6 except for the 4-bp insertion, do not induce gynogenesis. This diagnostic indel is also absent in the lines Mo1W and TX303 used as non-inducer controls in a GWAS study of *gim1/qhir1* (Hu *et al*, 2016). This later result highlights the preponderant role of the sub-region *qhir11* (containing *NLD*) compared to the sub-region *qhir12* (downstream of *NLD*) in haploid induction phenotype.

The fact that the right border of the interval is at a much larger physical distance from *NLD* is readily explained by important structural differences of PK6 with DH99 and B73. The presence of 70 kb (PK6) or 156 kb (B73) of unrelated sequence just downstream of *NLD* prevents genetic recombination in this region, which is reestablished further downstream in the region with synteny between the genomes. Similarly, structural differences upstream of *NLD* push the next recombination breakpoints at a distance of more than 62 kb in the B73 reference genome. The presence of a *NLD*-related pseudogene (GRMZM2G062313 annotated as lncRNA) in the B73 genome, which is absent from the PK6 sequence in this region, may be interpreted either as a possible cause for genome rearrangements in this region or as a consequence of inherent genome instability. The fact that the *NLD* gene is the only conserved genetic entity in this otherwise heavily rearranged region hints at a vital role in maize reproduction. Very recently, similar conclusions were reached by two independent studies (Kelliher *et al*, 2017; Liu *et al*, 2017).

Possible roles of *NLD* phospholipase in fertilization

Several lines of evidence indicate that *NLD* is a membrane-localized phospholipase necessary for successful fertilization: (i) the *NLD* promoter is strongly active in mature pollen and growing pollen tubes, (ii) the *NLD* protein is found in sperm cell plasma

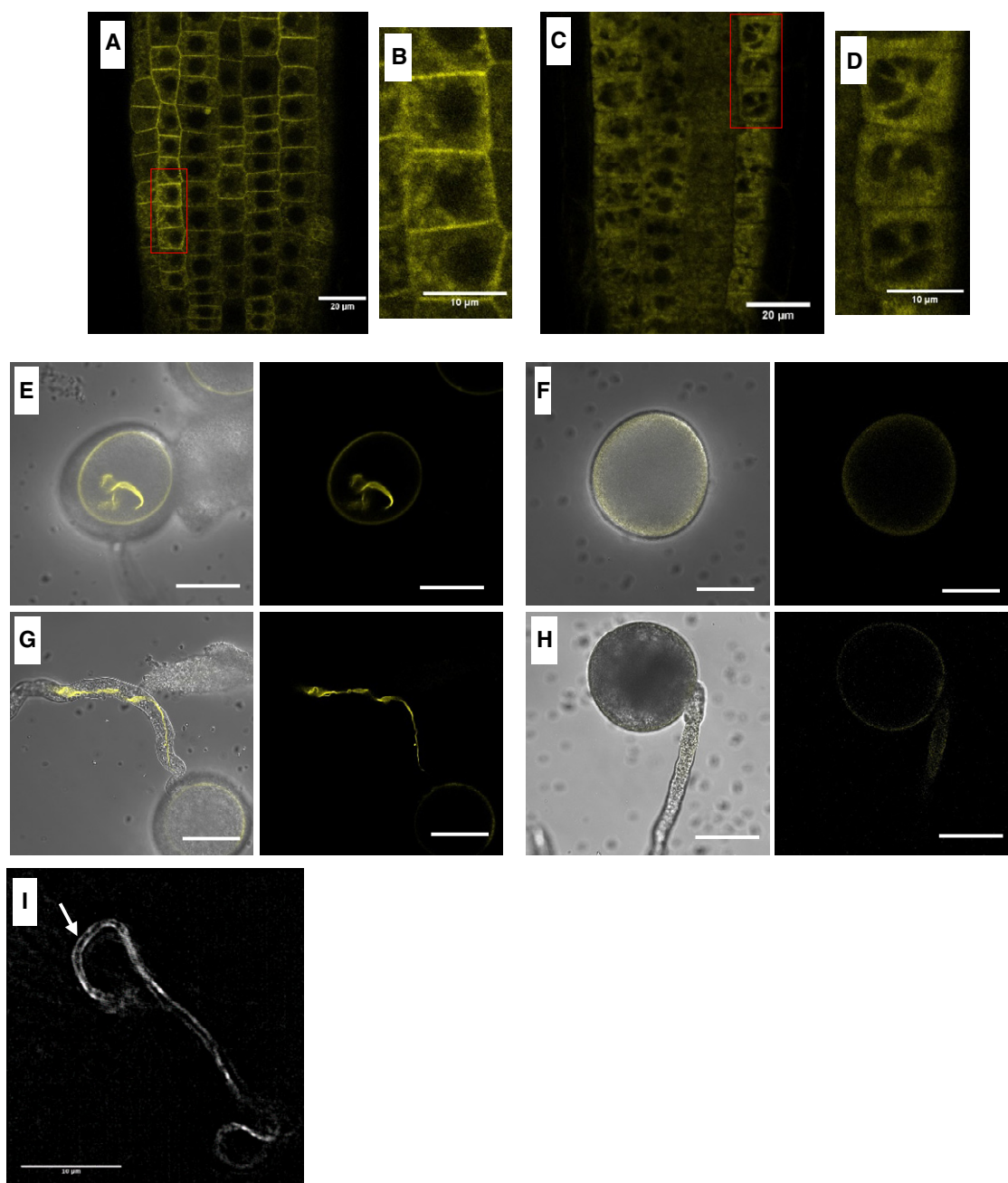


Figure 3. Subcellular localization of wild-type and truncated NLD protein.

- A–D Confocal imaging of *Arabidopsis* root tips expressing either wild-type NLD (A, B) or truncated NLD-PK6 (C, D) fused to citrine fluorescent protein. Panels (B) and (D) are zooms of the red frames represented in (A) and (C), respectively. Signal of both protein fusions can be seen in the cytoplasm and endomembrane compartments, whereas only wild-type NLD::citrine accumulated in the plasma membrane.
- E–H Confocal imaging of pollen grain (E, F) or pollen tube (G, H) expressing either wild-type NLD (E, G) or truncated NLD-PK6 (F, H) fused to citrine fluorescent protein, under the control of the *NLD* promoter. Scale bars, 50 μm . Bright field merged to fluorescence channels are represented on the left, whereas fluorescence channel is represented on the right.
- I Structured illumination microscopy (SIM) image (single image) of the male germ unit showing plasma membrane localization of wild-type NLD::citrine protein in sperm cells (arrow); scale bar, 10 μm .

membranes, (iii) a 4-bp insertion in the *NLD* coding sequence promotes the formation of haploid embryos, (iv) the localization of NLD to the plasma membrane is lost by the truncated protein predicted in the inducer lines, and (v) its annotation as a patatin-like

phospholipase A suggests that NLD is implicated in membrane lipid homeostasis of the sperm cells. Either a structural role in the maintenance of membrane integrity or a signaling role in the generation of signaling precursor from the membrane may be hypothesized.

Due to the broad functions attributed to patatin-like phospholipases (Scherer *et al*, 2010; Murakami *et al*, 2011), the molecular and cellular mechanisms linking NLD phospholipase activity to haploid induction require further investigations, which are not facilitated by the low penetrance of the induction phenotype (6–10%). Based on the loss of plasma membrane localization of the truncated NLD protein present in haploid inducer genotypes, one may speculate that defects in sperm cell/female gamete fusion could be at the origin of haploid induction. This scenario allows the connection of haploid induction and seed abortion to the same molecular mechanism (Xu *et al*, 2013). Indeed, haploid induction and seed abortion may reflect fusion failure of a sperm cell/egg cell and sperm cell/central cell, respectively. In *Arabidopsis*, RNAi knockdown of three pollen expressed *PLA2* genes that do not code for patatin-like PLA leads to developmental defects of pollen and results in reduced pollen fertility. However, the cellular and molecular mechanisms involved remain unrevealed (Kim *et al*, 2011).

Biotechnological applications

Haploid induction represents the initial step in the production of doubled haploid plants characterized by complete genome homozygosity (Geiger & Gordillo, 2009). Doubled haploids are an important breeding tool since they rapidly fix novel genome combinations. In maize, haploid inducers represent an inexpensive *in planta* system that does not require labor-intensive *in vitro* steps (Dwivedi *et al*, 2015). An alternative *in planta* strategy based on genetic engineering of CENTROMERIC HISTONE3 (CENH3) has recently been transferred from *Arabidopsis* (Ravi & Chan, 2010) to maize (Kelliher *et al*, 2016). It has the advantage to induce both maternal and paternal haploids, but the maternal haploid induction rate (< 1%) is far below the rate obtained using high inducer lines derived from stock 6 (~10%). The identification of *NLD* as the gene responsible for *in planta* haploid induction in maize opens new possibilities for (i) improvement of haploid induction rate in maize and (ii) translation of this breeding tool to other species. In maize, new alleles can be obtained by TILLING or by targeted mutagenesis with CRISPR-Cas9 (Barrangou & Doudna, 2016). Translation to other crops will first require the identification of functional orthologs, which is likely straightforward in monocots but complicated by the presence of numerous co-orthologs in dicots (Appendix Fig S2). The use of additional criteria such as expression in sperm cell or the presence of lipid anchors may facilitate the selection of candidate genes. Beyond *NLD*, it needs to be recalled that *gim1/qhir1* represents the predominant but not the only QTL (Barret *et al*, 2008; Prigge *et al*, 2012). The existence of at least seven other paternal QTL underlines the necessity to discover other molecular components. Putting together the different pieces of the puzzle will be valuable to better understand the double fertilization process, to improve doubled haploid production in maize and to extend the technology to other crops.

Materials and Methods

Plant material

Zea mays (maize) plants were either cultivated in fields located in Lyon (France) and Saint Martin de Hinx (France) or grown in

a S2 greenhouse with a 16-h illumination period (100 W m^{-2}) at 24/19°C (day/night) and without control of the relative humidity (Javelle *et al*, 2010). The derivation of highly recombinant inbred lines from a PK6 × DH99 cross has been described earlier (Barret *et al*, 2008). The public inbred lines of the core diversity panel were a gift from A. Charcosset (Camus-Kulandaivelu *et al*, 2006).

Score of haploid induction

The lines to be tested for haploid induction were crossed as male parent with hybrid F564 × DH7 homozygous for the *glossy1* mutation and/or hybrid PDH3 × PDH8 homozygous for the *liguleless* mutation (Melchinger *et al*, 2013). A minimum of 100 kernels was germinated and scored for the *glossy* (bright leaf surface, adhering water droplets) or *liguleless* (absence of ligule at the sheath/blade junction) phenotype indicative of haploid plantlets. Haploid induction rate was determined as the percentage of *glossy* or *liguleless* plantlets among germinated plantlets. Haploid state of *glossy* and *liguleless* plantlets screened were double-checked by genotyping polymorphic markers between the hybrid parents. Only *glossy* and *liguleless* plantlets with all markers at homozygous state were scored as haploid.

Segregation distortion

Heterozygous plants with a recombinant and a non-recombinant allele between markers GRMZM2G305400b_2 and SYN20148 (Appendix Table S1) were selfed. For each plant, 48 kernels, or less if the selfing was not successful, were sown and plantlets genotyped with 16 markers as indicated in Appendix Table S1. For each marker, the segregation distortion was expressed as the number of PK6 alleles divided by the number of DH99 alleles.

Genotyping

Two leaf punches (diameter 6 mm) were collected in plates with 96 deep wells and ground with two metallic balls (diameter 4 mm) in a 2010 Geno/Grinder® (SPEX Sample Prep, Stanmore, UK). Genomic DNA was extracted using a CTAB type lysis buffer and a magnetic bead based purification. KASP markers were designed and genotyped in 1,536-well microtiter plates as described (He *et al*, 2014) using a Meridian dispenser (LGC Standards, Molsheim, France), a Fusion3™ laser plate sealer (LGC Standards, Molsheim, France), a Hydrocycler™ thermal cycler (LGC Standards, Molsheim, France), and a PHERAstar FS HTS microplate reader (BMG Labtech, Champigny-sur-Marne, France).

BAC library screen

The BAC libraries Zma-B-PK6 (HindIII) and Zma-B-PK6e (EcoRI) were constructed in the pIndigoBAC5 vector in strain DH10B-T1R and screened as described (Chalhoub *et al*, 2004). Selected BAC clones were sequenced with a PacBio RS II long read sequencer (Pacific Biosciences). Sequence reads were assembled with the SMRT Analysis software (version 2.3.0) and the resulting consensus sequence was given the GenBank accession number KX852318.

Phylogenetic analysis

A complete list of putative orthologs of *Arabidopsis* patatin-like PLs was obtained by BLAST search of the maize RefGen_v3 database (<http://www.maizegdb.org/>). The predicted amino acid sequences were aligned using MUSCLE in the SEAVIEW program (Gouy *et al*, 2010). Well-aligned amino acid blocks were selected for phylogenetic analyses using Gblocks. Maximum-likelihood phylogenetic analyses were performed in PhyML (Guindon *et al*, 2009) incorporating 1,000 bootstrap replicates and using LG substitution models for amino acid sequence data.

Prediction of lipid modification sites

For the prediction of lipid modification sites, the function “Search for palmitoylation, *N*-myristoylation, farnesylation geranylgeranylation post-translational modifications” and the threshold setting “High” of the “GPS-lipid” web site predictor (Xie *et al*, 2016) were used.

RNA extraction, reverse transcription, and PCR

For RNA extraction from pollen grain, a CTAB method was used (Messias *et al*, 2014). For others tissues, fresh tissue was quick frozen in liquid nitrogen and ground to powder either with a Qiagen Mixer-Mill 3MM or with a mortar and pestle. Approximately 100 mg was used for RNA extraction with TRI reagent (Molecular Research Center), DNase treatment with RNase-free DNase (Ambion), and reverse transcription using reverse transcriptase without RNaseH activity (Fermentas) according to the instructions of the suppliers. RT-PCR and qRT-PCR were carried out and relative expression levels calculated by the $\Delta\Delta C_T$ method as described (Javelle *et al*, 2010).

Constructs for plant transformation

The plasmids used for the production of *NLD_{DH99}*-OE plants contained the backbone of vector pSB11 (Ishida *et al*, 1996), a Basta resistance cassette (*Oryza sativa* (rice) *Actin* promoter and intron, *Bar* gene, and *Nos* terminator) next to the right border, a GFP cassette (*CsVMV* promoter and *FAD2* intron, *GFP* gene, and *Nos* terminator), and the *NLD_{DH99}* genomic coding sequence (primers PLPK6_HDPK_F1 and PLPK6_HD99_R1; see Appendix Table S4) under the control of the constitutive rice *Actin* promoter and intron.

A *NLD* upstream fragment of line B73 (containing 2,657 bp upstream of the ATG start codon) was amplified with the primer pair attB4_prom_PL_2576_B73/attB1r_prom_PL_2576_B73 and recombined into pENTR P4-P1R (Invitrogen) by BP reaction (*pNLD*). Transcriptional fusions to the *GUS* reporter gene were obtained by triple LR reaction between the putative promoter fragment, the *GUS* gene from pEN-L1-SI-L2, pmock3', and the destination vector pBb7m34GW (Karimi *et al*, 2007).

NLD-CDS (without STOP codon) from DH99 and PK6 was amplified from mature anther cDNA with primer pairs PL-CDS-F_Dtopo/PL-CDS-HD99-R2 and PL-CDS-F_Dtopo/PL-CDS-PK6-R2, respectively, and cloned into pENTR/D-topo (Invitrogen). The cassettes pUBQ10::*NLD*-CDS::CITRINE were obtained by triple LR reaction with the fragments pUBQ10 and mCITRINE (Jaillais *et al*, 2011) into the destination vector pK7m34GW (Karimi *et al*, 2005). The cassettes *pNLD*::*NLD*-CDS::CITRINE were obtained by triple LR

reaction with the fragments pNLD and mCITRINE (Jaillais *et al*, 2011) into the destination vector pBb7m34GW (Karimi *et al*, 2007).

Plant transformation

Agrobacterium-mediated transformation of maize inbred line A188 was executed according to a published protocol (Ishida *et al*, 1996). For each transformation event, the number of T-DNA insertions was evaluated by qPCR, and the integrity of the transgene was verified by PCR with primers situated in the *AtSac66* terminator downstream of the construct of interest next to the left border.

Arabidopsis ecotype Col-0 was transformed with the floral-dip method (Clough and Bent, 1998) and a modified procedure for *Agrobacterium* preparation (Logemann *et al*, 2006).

Microscopy

Fluorescence of transgenic plants harboring the citrine reporter was detected with a Zeiss LSM 710 Laser Scanning Microscope using 520- to 580-nm bandpass filters after excitation at 510 nm. Image data were analyzed with ImageJ software. For SIM, pollen was imaged with a 63× Apo NA 1.4 oil objective of a Zeiss Elyra PS1 system equipped with 488-nm laser and 495- to 575-nm band pass filter. Image stacks were taken with 0.125-μm z-steps and 15 images per section (five phases and three angles). Images were then processed using Zen software using following parameters: manual processing, baseline-shifted, auto-noise filtering, and 1.5 weighting.

Histochemical detection of GUS activity

GUS (beta-glucuronidase) histochemical staining was performed by dipping tissues into the following solution: 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), 0.05% Triton X-100, 100 mM sodium phosphate (pH 7), and 0.5 mM potassium ferrocyanure and 0.5 mM potassium ferricyanure. The enzymatic reaction was performed overnight at 37°C after vacuum infiltration.

Expanded View for this article is available online.

Acknowledgements

We thank Alexis Lacroix, Yannick Rasmus, Priscilla Angelot, and Justin Berger for maize culture; Hervé Leyral and Isabelle Desbouchages for media preparation; and Frédérique Rozier, Annick Dedieu, Véronique Boltz, Pierre Chambrier, Wiebke Bretting, and Erika Consoli for technical assistance. We are grateful to Gwyneth Ingram for thoughtful discussions. We also acknowledge the contribution of the INRA field station in Saint Martin de Hinx (Carine Palaffre), the greenhouse technical platform of FR3728 BioEnviS at Université Lyon 1, and the AG platform (Bariza Blanquier) as well as the PLATIM imaging facility of the SFR Biosciences Gerland-Lyon Sud (UMS344/US8). Funding for this work has been refused twice by the French granting agency ANR. ASK was supported by a fellowship of the Egyptian Government and LMG by a CIFRE fellowship of the ANRT (grant N° 2015/0777).

Author contributions

PMR and TW were involved in conceptualization; JPM, JC, HB, PMR, and TW contributed to methodology; LMG, AK, JBL, SC, GG, JL, GB, VB, PMR, and TW performed investigation; HB, PB, JC, and JPM provided resources; LMG, PMR, and TW were involved in writing the original draft as well as review and

editing; PMR, JPM, HB, and TW were involved in supervision; JPM, PMR and TW were involved in project administration and obtained funding acquisition.

Conflict of interest

LMG, JBL, JC and JPM are employees of LIMAGRAIN Europe. Pending patent applications PCT/EP2016/060202 (published as WO2016177887), EP3091076.

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